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PATENT APPLICATION
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Application No.: 08/523,004 Group Art Unit: 164

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For: NOVEL MUCOSAL VASCULAR ADDRESSINS

CERTIFICATE OF MAILING

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DECLARATION UNDER 37 C.F.R. § 1.132

Assistant Commissioner for Patents
Washington, DC 20231

Sir:

I, Michael J. Briskin, Ph.D., of 28 Harbell Street, Lexington, Massachusetts 02421, hereby declare and state that:

1. I received a Bachelor of Science in Biology in 1979 from The University of California, Los Angeles, and a doctorate in Molecular Biology in 1988 from the University of California, Los Angeles.

2. In 1993, upon completion of my postdoctoral research at Amgen, Inc. and Stanford University School of Medicine, Department of Pathology, I accepted a position at LeukoSite, Inc. I am presently employed as a Senior Research Scientist by LeukoSite, Inc., Assignee of the subject application.

3. I am familiar with the subject application and the invention claimed therein.

4. Since the filing of the subject application, additional studies of human MAdCAM-1 have been completed at LeukoSite, which support the subject application. In particular, a series of point mutations in the human MAdCAM-1 amino acid sequence were prepared. The additional work described herein was performed by Nancy Green and Josh Rosebrook, employees of LeukoSite, Inc., working under my direction and supervision.

5. As described herein, structure/function studies of human MAdCAM-1 have been carried out. Variants of human MAdCAM-1 were generated in order to define sites required for binding to the $\alpha 4\beta 7$ integrin. 31 different point mutants of human MAdCAM-1 were produced. The function of the resulting variants was assessed using an adhesion assay which monitors binding to the $\alpha 4\beta 7$ integrin. 14 of the 31 point mutants assessed displayed mean binding to $\alpha 4\beta 7$ at levels between 80% - 100% of the control in the presence of Mn^{2+} (The Table).

Mutagenesis

Human MAdCAM-1 mutants were prepared as soluble immunoglobulin fusion proteins (also referred to as "HuMAdIg receptor" proteins) consisting of the entire extracellular domain of human MAdCAM-1 fused to the entire constant region of an Fc mutated human IgG1 heavy chain (Tidswell, M., *et al., J. Immunol., 159*:1497-1505 (1997)). A series of point mutants of a construct encoding a soluble human MAdCAM-1-Ig fusion protein (a wild type HuMAdIg receptor) were made using the QuikChange Mutagenesis Kit and P*fu* DNA polymerase (Stratagene, La Jolla, CA). Alanine was substituted in all cases except for additional CD Loop mutations, L45R and D46E. Mutagenic primers contained 10-16 bp of template sequence adjacent to the changed bases

(e.g., the sense primer for D46A reads 5'-CGG GGC CTG GcC ACC AGC CTG G-3'). All mutated regions were sequenced using the ThermoSequenase Kit (Amersham Life Science, Inc., Cleveland, OH).

Preparation of Fusion Proteins

Soluble chimeric proteins were produced by transient transfection of CHO/P cells. CHO/P cells were transfected with constructs encoding mutant MAdCAM-1-Ig fusions proteins. CHO/P cells were plated at 1 x10^6 cells/10 cm plate (Falcon), and incubated overnight at 37°C. Media was removed and cells were rinsed with 2 ml Opti-MEM-1 (GIBCO, Life Technologies, Gaithersburg, MD). LIPOFECTAMINE Reagent (GIBCO) (60 μl/plate) was mixed with 10 μg plasmid DNA in 1.6 mls Opti-MEM-1, incubated 25 minutes at ambient temperature and subsequently added to cells with 6.4 ml additional Opti-MEM-1. After 2.25 hours incubation at 37°C, an equal volume (8 mls) of alpha-MEM/20% fetal bovine serum (GIBCO) was added to cells for overnight incubation. Approximately 18 hours after transfection, transfection media was replaced with 10 mls of MEM-α media and then incubated 72-96 hours before collection for purification. Chromatography columns (BioRad) were packed with 3 mls of Protein A Sepharose Fast Flow resin (Pharmacia Biotech) and pre-equilibrated with phosphate buffered saline (PBS). Mutant Ig chimera supernatanants were diluted 1:1 in PBS, filtered and applied to columns by gravity flow. Columns were washed with 60 mls PBS, then material eluted by addition of 4 ml of 0.1 M Citrate pH 3.5, neutralized and dialyzed against PBS.

Soluble proteins were quantitated by ELISA. 96-well plates (NUNC Maxisorb) were pre-coated with anti-human IgG (Jackson Immuno-Research, West Grove, PA), 10 μg/ml, 50 μl/well in carbonate buffer pH 9.5 and incubated overnight at 4°C, then blocked with PBS/5% gelatin for 2 hours at 37°C. Samples and standard (human IgG1) were diluted in 1X THST (1mM glycine, 0.5 M NaCl, 50 mM TRIZMA base pH 8.0, 0.05% TWEEN 20), applied to plates, and incubated for 2 hours at room temperature. Plates were rinsed with 1X THST. Detection was via peroxidase-conjugated Goat-anti mouse IgG (Jackson), 1:4000 in 1X THST, 50 μl/well. Assays were visualized with OPD (Sigma) and absorbance was read at 490 nm.

Adhesion Assays

Cell lines used for functional adhesion assays were CHO/P (Heffernan, M. and Dennis, J.D., *Nucl. Acids Res., 19*:85 (1991)), RPMI 8866 which only express α4β7 (Erle, D.J., *et al., J. Immunol., 153*:517-528 (1994)), and Ramos which express only α4β1 (ATCC). For adhesion assays with soluble huMAdIg receptors, proteins were plated onto wells of a 96-well RIA plate (Costar, Cambridge, MA) at 0.2 μg/ml in 50 μl phosphate buffered saline. Plates were incubated overnight a 4°C, and blocked for 2 hours at 37°C (PBS, 10% calf serum, Gibco). Labeled RPMI 8866 cells were added to each well at 1.25 x 10^6/well, then plates were incubated for 30 minutes at room temperature. Plates were washed for 2 cycles on a Microplate Autowasher (Biotek Instruments), then fluorescence was quantitated. Each sample was assayed in triplicate and all assays were performed at least three times.

Results

Mutagenesis of Individual Human MAdCAM-1 Residues

In order to further define the α4β7 binding site in human MAdCAM-,1 a series of single amino acid substitutions in regions that were predicted to be important for IgCAM/integrin interactions based on other studies, were generated. The study focused primarily on selected residues in interstrand loops in both domains 1 and 2 of human MAdCAM. The approximate locations of these loops were based upon the crystal structure of human VCAM-1. The locations that were targeted aligned with interchain loops of other IgCAMs that have been shown to be important in integrin recognition, namely the CD and EF loops of domain 1 and the C'E and FG loops of domain 2 (Wang, J-H., *et al., Proc. Natl. Acad. Sci. USA, 92*:5714-5718 (1995); Jones, E.Y., *et al., Nature, 373*:539-544 (1995)). Additionally, some of these regions were shown to be important for ICAM-1, ICAM-3 and VCAM-1 mediated adhesion (Staunton, D.E., *et al., Cell, 61*:243 (1990); Osborn, L., *et al., J. Cell Biol., 124*:601 (1994); Holness, C.A., *et al., J. Biol. Chem., 270*:877 (1995)). In most instances, alanine substitution was used, and most amino acids in each region were targeted for mutation with the exception of small, hydrophobic resides. As noted above, human MAdCAM-1 mutants were prepared as soluble Ig-fusion proteins consisting of the entire extracellular domain of human MAdCAM-1 fused to the

entire constant region of an Fc mutated human IgG1 heavy chain (Tidswell, M., *et al.*, *J. Immunol.*, *157*:1497-1505 (1997)). Recombinant soluble huMAdIg receptor proteins were transiently produced in CHO/P cells, purified by protein A chromatography, and quantitated in an IgG based ELISA to ensure that similar amounts of receptors were used in each experiment.

Adhesion was compared to binding to wild type MAdCAM-Ig fusion protein in an RPMI 8866 cell binding assay, initially in the absence of activating stimuli. Alanine substitution of R39, L41, D42, T43, R70, E148, E151, E152, E157 and H195 reduced adhesion to less than 25% adhesion as compared to adhesion of wild-type MAdCAM-1 (the Table). Additionally, a conservative change of D42E essentially abolished binding as adhesion was reduced to only 4.3% of normal. Moderate effects (25-50% adhesion) on binding were observed with S55 and E149, while mild effects (50-75% adhesion) were seen with L64, E145, Q147 and D156. All other mutations did not affect binding significantly (75-100% adhesion), with the exception of alanine substitution of R60 and E192 which resulted in significant increases in binding (the Table).

To further assess the severity of the mutations tested, the assays were repeated in the presence of 1 mM Mn^{2+}, which has been shown to fully activate integrins, including $\alpha 4\beta 7$ (Berlin, C., *et al.*, *Cell, 74*:185-195 (1993); Briskin, M.J., *et al.*, *J. Immunol.*, *156*:719-726 (1996)). Under these conditions, mutation of only three residues (L41, D42 and E148) yielded mutants which display adhesion at levels less than 25% of the adhesion of wild type MAdCAM-1. Three other residues, R70, E152 and E157 were also moderately (25%-50% adhesion) affected in the presence of Mn^{2+}. Additionally, the two mutations (R60 and E192) that originally showed increased binding displayed binding at similar levels to native MAdCAM-1 under these conditions.

The results of performing the assays with activated integrin were similar to looking at adhesion of the mutant receptors at higher concentrations. The severe mutations such as L41A and D42A or E could not be rescued by using higher receptor densities, while the R70A mutation was only partially rescued at receptor concentrations 4-fold higher than in the standard assays. Conversely, many of the mutations in the DE loop of domain 2 were restored to levels closer to native MAdCAM at higher receptor avidity, indicating that these mutations might have different mechanistic effects.

Discussion

Site-directed mutagenesis was performed to define critical α4β7 binding residues in both Ig domains 1 and 2. The results are consistent with data obtained for other Ig-CAMS showing that an essential contact site resides in the CD loop in domain 1, the most significant residues being L41 and D42 (the Table). Alanine substitution in either of these sites abolishes integrin interactions, even when α4β7 is fully activated by Mn^{2+}. The absolute requirement for a defined structure of the CD loop is illustrated by the fact that a conservative substitution, D42E, also results in a complete abrogation of binding (without Mn^{2+}). Additionally, we also found that R39A resulted in significantly decreased adhesion, while the analogous residue in murine MAdCAM-1 R38 was unaffected by mutation to alanine (Viney, J.L., *et al.*, *J. Immunol.*, *157*:2488-2497 (1997)). The discrepancy between this data for human R39A versus murine R38A MAdCAM-1 may reflect a technical difference in the assays performed or may reflect structural distinctions due to the weak sequence conservation (57% identity) between the murine and human homologs (Shyjan, A.M., *et al.*, *J. Immunol.*, *156*:2851-2857 (1996)).

TABLE

Mutant	without Mn++ Mean	SDEV	with Mn++ Mean	SDEV
R39A	20.48	8.24	62.25	3.14
L41A	0.25	0.50	10.88	3.39
D42A	1.38	2.75	1.5	1.24
D42E	4.3	8.53	23.1	5.08
T43A	5.08	4.44	54.4	3.56
S55A	27.94	25.21	88.65	5.17
R60A	224.68	27.10	97.4	5.89
N61A	118.23	38.56	88.2	6.23
L64A	59 8	19.11	81.9	3.20
S65A	101.2	31.20	84.4	4.30
T69A	144.73	38.07	83.93	7.25
R70A	1.05	1.22	48.4	4.3
P144A	102.93	17.12	80.23	8.55
E145A	67.83	30.35	80.7	6.73
Q147A	73.8	14.00	71.5	6.8
E148A	1.43	1.67	22.6	5.05
E149A	37.9	23.80	62.9	9.20
E150A	116.85	23.05	82.58	2.22
E151A	6.18	7.15	67.3	0.42
E152A	0.8	0.98	29.2	6.08
P153A	88.38	38.41	86.73	8.92
Q154A	91.04	34.00	72.1	9.10
D156A	56.08	6.78	65.78	####
E157A	8.6	5.27	49.15	####
D158A	86.5	11.90	83.68	2.74
R162A	97.18	15.60	75.48	6.16
R187	92.83	17.33	85.23	3.54
E192A	147.95	4.14	85.65	3.80
S194A	74.55	28.24	80.15	5.49
H195A	22.45	10.31	66.78	6.88
H196A	86.03	15.91	71.43	4.57

I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true. Moreover, these statements were made with the knowledge that willful false statements and the like made by me are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Michael J. Briskin

11-30-98

Date